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In Vitro Gene Delivery to Hepatocytes with Galactosylated Polyethylenimine

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A hepatocyte-directed vector has been developed; it includes several key features thought to favor *in vivo* gene delivery to the liver: electrostatically neutral particles which avoid nonspecific binding to other cells, to the extracellular matrix, and to complement proteins; asialoglycoprotein receptor-mediated endocytosis which may address the complexes to the perinuclear region; and polyethylenimine (PEI)-mediated endosome buffering and swelling as an escape mechanism to the cytoplasm. This system is based on a 5% galactose-bearing polyethylenimine (PEI-gal) polymer which is condensed with plasmid DNA to neutrality. Murine (BNL CL.2) and human (HepG2) hepatocyte-derived cell lines were transfected 10^4 – 10^5 -fold more efficiently than murine fibroblasts (3T3), whether transfection was assessed globally (luciferase expression from the cell extract) or following histochemical staining (β -galactosidase). Under these conditions, over 50% of the hepatocytes were selectively transfected in the presence of 10% serum. Transfection was suppressed by removal of the targeting galactose residues, by their replacement with glucose, or by the addition of excess asialofetuin. Thus, results from comparative and competitive experiments indicate the asialoglycoprotein receptor is involved in transfection of hepatocytes with neutral PEI-gal/DNA complexes.

In vivo gene delivery with nonviral vectors has been demonstrated in many cases and provides the basis for several clinical trials (Nabel, G. J., et al., 1993; Caplen et al., 1994; Nabel, E. G., et al., 1994; Crystal, 1995). However, with some exceptions (Stribling et al., 1992; Logan et al., 1995), efficiency, especially when it comes to the percentage and type of transfected cells, has been rather low (Perales et al., 1994; Thierry et al., 1995; Tsukamoto et al., 1995; McLachlan et al., 1995; Schwartz et al., 1995; Noguiezhellin et al., 1996; Stephan et al., 1996; Lee et al., 1996). Most work has been done with cationic lipid/plasmid complexes. Their net ionic charge (i.e. the ratio of cationic vector over anionic phosphate) governs the fate of the resulting particles (Behr et al., 1989; Schwartz et al., 1995; Remy et al., 1995) and leads to a dilemma. Transfection is most efficient only when particles are cationic and thus can be taken up by adherent cells following binding to anionic cell surface proteoglycans (Mislick and Baldeschwieler, 1996; Labat-Moleur et al., 1996); they will however bind to quite similar polyanionic glycans present in the extracellular matrix. Conversely, anionic particles will not be retained, but will not bind efficiently to the cell surface either. The tricky choice of the cationic vector/DNA ratio is further complicated by the fact that around neutrality particles become generally much larger and are unstable (Jaaskelainen et al., 1994); indeed, particle size is an important concern too when considering diffusion within a tissue or escape from the reticuloendothelial system. A way out of this dilemma is development of neutral particles supplemented with a ligand which will prevent complex aggregation and allow cell binding (Remy et al., 1995).

In the pioneering work by Wu and Wu (1987) on hepatocyte targeting with asialoglycoprotein-polylysine/

plasmid complexes, most components for dealing with these problems were empirically present. Calculations with the amounts of cationic vector and DNA they used show that the charge of the complexes was on the anionic side, leading to particles which were stable and diffusible *in vivo*. Efforts in downsizing the particles were undertaken by complex formation in a decreasing salt gradient. Finally, cell binding by the anionic complexes was due to the asialoglycoprotein component. Transgene expression was achieved *in vivo* (Wu et al., 1989, 1991; Wilson et al., 1992; Stankovics et al., 1994; Frese et al., 1994), yet most of the DNA remained for days in intracellular vacuoles (Chowdhury et al., 1993). *In vitro* work showed that these complexes required the lysosomotropic weak base chloroquine to be efficiently released into the cytoplasm (Plank et al., 1992; Midoux et al., 1993; Wadhwa et al., 1995); ip injection of chloroquine prior to gene delivery *in vivo* was even attempted (Merwin et al., 1994).

We recently described a new property for an old compound (Boussif et al., 1995, 1996). Indeed, the versatile cationic polymer polyethylenimine (PEI) was found to be among the most efficient polycationic gene transfer vectors to date. PEI shares endosome buffering capacity with chloroquine, and this "proton sponge" effect provides the basis for endosomal escape for the PEI/DNA complexes. On the other hand, chemical adduction to the ethylenimine network does not seem to interfere with gene delivery (Remy et al., 1997). Therefore, PEI constitutes *per se* an outstanding core for the design of more sophisticated devices. Here, we demonstrate that neutral PEI/DNA complexes bearing galactose residues transfect efficiently and specifically hepatocyte-derived cell lines in the presence of serum and in the absence of any extra membrane-disrupting agent.

EXPERIMENTAL PROCEDURES

Chemicals. Polyethylenimine (25 kDa average M_w), lactose, maltose, and sodium cyanoborohydride were purchased from Aldrich (Saint Quentin-Fallavier, France). Asialofetuin and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, MO).

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Glycosylation of Polyethylenimine. PEI (31 mg; 581 μ mol of amine functions assuming a mean M_w of 43 Da for the repeating unit) and the disaccharides lactose or maltose (11 mg, 30 μ mol, M_w = 360.3 Da) were dissolved in 1 mL of sodium borate buffer (200 mM, pH 8.5). After addition of sodium cyanoborohydride (5 equiv/disaccharide), the reaction mixture was incubated for 48 h at 40 °C in a water bath. After cooling to room temperature, the polymeric material was purified by ultrafiltration through an Amicon membrane (cutoff, 30 000 Da, Millipore) and washing with distilled water. Drying of the residue *in vacuo* led to 36 mg of glycosylated polyethylenimine. Carbohydrate quantitation with the resorcinol test led to 5% conjugation. *N/P* calculations for PEI-gal and PEI-glu (see below) were thus based on a mean M_w of 61 Da.

Cell Lines and Cell Culture. NIH 3T3 murine fibroblasts and HepG2 human hepatocarcinoma cell lines were purchased from ATCC (Rockville, MD) and grown in Dulbecco's modified Eagle medium (DMEM). BNL CL2 murine hepatocytes were kindly provided by E. Wagner (Bender Co., Wien, Austria) and grown in high-glucose DMEM (4.5 g/L). All cell culture media were supplemented with 10% FCS (fetal calf serum, D. Dutcher, Brumath, France), 2 mM L-glutamine (Gibco BRL, Cergy-Pontoise, France), 100 units/mL penicillin (Gibco BRL), and 100 μ g/mL streptomycin (Gibco BRL). Cells were maintained at 37 °C in a 5% CO₂ humidified atmosphere. When 80% confluent, they were detached with saline trypsin/EDTA (Gibco, BRL) and grown in new flasks at a 1/10 dilution.

Plasmids. pCMV-luc and pCMV-NLSlacZ, encoding respectively the *Photinus pyralis* luciferase and the bacterial β -galactosidase genes under the control of the cytomegalovirus enhancer/promoter, were kindly provided by M. Scarpa (CRIBI, Padova, Italy). Plasmids were purified from *Escherichia coli* strain XL blue using Qiagen columns (Courtaboeuf, France).

Cell Transfection. Adherent cells were seeded in 24-well plates (Costar, D. Dutcher) the day before transfection so they could reach 60–70% confluence during transfection. All experiments were done in triplicate. Prior to transfection, cells were rinsed and 1 mL of culture medium complemented with 10% FCS was added. Two micrograms of the desired plasmid (from a ca. 1.5 mg/mL solution in 10 mM Tris/1 mM EDTA buffer at pH 7.4) was diluted into 50 μ L of 0.15 M NaCl. The desired amount of 25 kDa PEI or glycosylated PEI [from 10 mM stock solutions of PEI, PEI-gal, or PEI-glu in water at pH 7.5; 1 *N/P* equivalent corresponds to the amount of polymer necessary to have one amino group (43 Da mean M_w for PEI and 61 Da for PEI derivatives) per phosphate of nucleic acid (330 Da mean M_w) (Boussif et al., 1996)] was diluted into 50 μ L of 0.15 M NaCl, vortexed gently, and spun down.

Fifteen minutes later, the cationic vector was added at once to the plasmid solution [and not the reverse order; see Boussif et al. (1996)], mixed, vortexed, and spun down. The amounts and volumes given above refer to those in a single well and were actually 3-fold larger and distributed in three wells. After ca. 10 min, the resulting mixture was added to the cells and the cell supernatant was uniformly distributed with a gentle horizontal hand rotation. Immediately after, the cell culture dish was centrifuged (Sigma 3K10, Bioblock) for 5 min at 1500 rpm (ca. 280g). The cells were cultured for 24 h and then tested for reporter gene expression.

Luciferase Assay. Luciferase gene expression was measured by luminescence. The culture medium was discarded and cell lysate harvested upon cell incubation

for 30 min at room temperature in Lysis Reagent 1 \times (Promega, Madison, WI). The lysate was vortexed gently and centrifuged for 5 min at 14 000 rpm (ca. 17530g) at 4 °C. Twenty microliters of lysate was diluted into 100 μ L of luciferase reaction buffer (Promega), and the luminescence was measured for 10 s (Biolumat LB 9500, Berthold, Wilbach, Germany). The results were expressed as light units per milligram of cell protein (BCA assay, Pierce).

X-Gal Staining. β -Galactosidase gene expression was detected by histochemical cell staining using X-Gal (Euromedex, Souffelweyersheim, France) as described (Boussif et al., 1996).

RESULTS

Synthesis and DNA Binding Properties of the PEI-gal Molecular Conjugate. Several types of ligands have been conjugated to polycations for transferring genes to hepatocytes following endocytosis *via* the asialoglycoprotein receptor: asialoorosomucoid, a natural protein ligand (Wu and Wu, 1987; Cristiano et al., 1993), multiantennary synthetic galactose derivatives (Remy et al., 1995; Plank et al., 1992; Wadhwa et al., 1995; Merwin et al., 1994), and simple galactose groups (Perales et al., 1994; Midoux et al., 1993; Chen et al., 1994; Martinezfong et al., 1994). We opted for the simplest solution as strong binding by individual residues may be unnecessary provided sufficient galactose residues are present on each PEI/DNA particle. The most straightforward way to link galactose to PEI is *via* imine formation of lactose (galactosyl- α 1,4-glucose, see Figure 1) with the amine functions of PEI, followed by *in situ* reduction back to a substituted amine (Martinezfong et al., 1994). This introduces a four-carbon hydrophilic spacer between the polymeric network and the galactose ligand.

The percentage of derivatization is an important factor to consider, too. A rough preliminary screening led to 5% of the total amine functions being a good choice. It should be noted that, in contrast to amide or thiourea linkers, the present sequence of chemical reactions does not decrease the original number of cationic charges borne by the polymer. Furthermore, in contrast to polylysine-based vectors, only a fraction (ca. 25%) of the amine functions of PEI are protonated at physiological pH (Boussif et al., 1995). Complex formation with anionic DNA is expected to increase this fraction, especially at a low PEI/DNA ratio. Some surface charge measurements (zetasizer, Malvern) confirmed this view since complexes were found to be neutral when PEI (nitrogen)/DNA (phosphate) *N/P* = 3. The ratio where DNA binding is complete was assessed by agarose gel electrophoresis of complexes formed between PEI or PEI-gal and plasmid DNA. As shown in Figure 2, all the DNA was retained for an *N/P* ratio of above 2, whether with the native or derivatized polymers. The best conditions for *in vivo* transfection would therefore be around *N/P* = 2–3.

Hepatocyte Transfection in Serum Remains Efficient at Low PEI-gal/DNA Charge Ratios. We recently worked out optimized conditions for transfection with lipopolyamines and PEI which tolerate the presence of serum (Boussif et al., 1996); these conditions have been used here to compare PEI to PEI-gal-mediated transfection for various cation/anion ratios. The pK_a 's of PEI being variable, this ratio was expressed as PEI amine monomer over DNA phosphate (*N/P*). A reasonable assumption is that, above DNA charge neutralization by PEI (*N/P* = 3), excess complexed PEI behaves as free PEI; i.e. every one out of four nitrogen atoms is protonated.

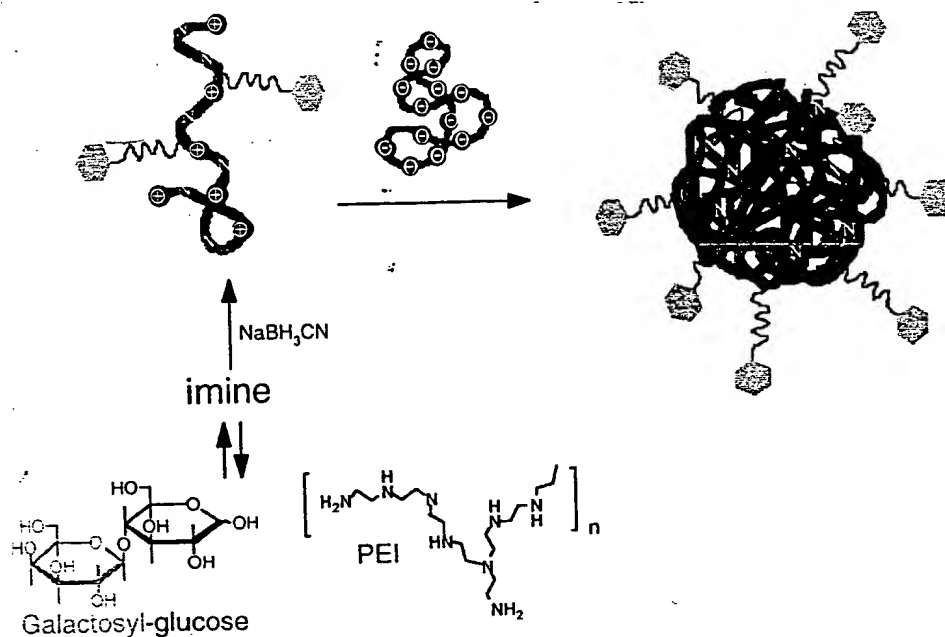


Figure 1. Idealized scheme for the conjugation of galactose to polyethylenimine via imine formation with lactose, and subsequent condensation of PEI-gal with plasmid DNA into neutral galactose-bearing complexes (objects are not to scale).

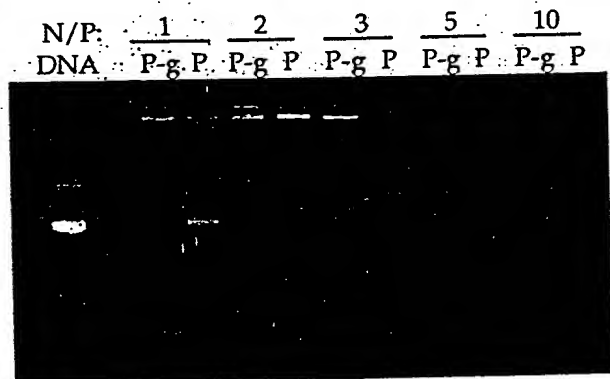


Figure 2. Analysis of complex formation by agarose gel electrophoresis. Above $N/P = 2$ (N/P is the molar ratio of PEI amine functions over DNA phosphates), all the DNA is complexed and remains in the well. pCMV-Luc plasmid DNA (2 μ g) was mixed with increasing amounts of PEI (lanes P) or of PEI-gal (lanes P-g) in a total volume of 100 μ L of 0.15 M NaCl as was performed for the transfection experiments (see Experimental Procedures). After 15 min, 10 μ L of each solution (200 ng/well) was loaded in a well for electrophoretic separation (0.8% agarose in 150 mM Tris-acetate buffer at pH 7; electrophoresis at 65 V for 90 min). DNA was visualized with ethidium bromide.

Adherent 3T3 murine fibroblasts, as expected, were efficiently transfected with cationic PEI/pCMV-Luc complexes ($N/P = 10$ –5, Figure 3a) which bind nonspecifically to anionic cell surface "receptors". Neutral or anionic complexes ($N/P = 3$ –1) which cannot bind efficiently to the cells displayed poor transfection ability. These results were confirmed histochemically by cell counting after pCMV-NLSLacZ transfection (ca. 80 and <0.1% transfected cells for $N/P = 10$ and 2, respectively; data not shown). Fibroblasts do not express [$\rho(-)$] the asialoglycoprotein receptor and are not expected to display enhanced PEI-gal-mediated gene delivery. In fact, galactose conjugation seemed even to interfere with the ionic cell binding process, as transfection ($N/P = 10$ and 5) decreased by 1–2 orders of magnitude.

Murine BNL CL.2 hepatocyte (Figure 3b) and human HepG2 hepatoma (Figure 3c) cell lines also were efficiently transfected by cationic PEI/DNA complexes [$>10^9$ LU/(mg of protein)] and showed similar trends toward poor transfection [10^4 – 10^5 LU/(mg of protein)] for

neutral complexes. Here however, galactose-bearing complexes retained high transfection levels down to below neutrality ($N/P = 3$ and 2). Decreasing the vector/plasmid ratio further ($N/P = 1$) led to a sharp efficiency decrease. Indeed, less DNA is complexed (Figure 2), fewer galactose residues are present on the complexes, and DNA may loop out of the anionic complexes and interfere with receptor binding.

Curves shown in panels a–c of Figure 3 are representative of six independent sets of experiments performed with two batches of PEI-gal, pCMV-Luc from several batches, and cells with various passage numbers. Absolute luciferase expression values stayed within 1 order of magnitude for ion-mediated PEI ($N/P = 10$) and galactose receptor-mediated PEI-gal ($N/P = 3$ and 2) transfections. Poor ion-mediated transfection values with neutral PEI/DNA complexes were more scattered (2 orders of magnitude). This had been noticed previously for cationic lipids (Remy et al., 1995) and may be attributed to the fact that neutral complexes are unstable (Jaaskelainen et al., 1994) and that a weak interaction with cells is prone to larger binding variations. Relative values taken from this large set of data however confirmed the conclusions derived above.

Neutral PEI-gal/DNA Complexes Show a 10^4 – 10^5 -Fold Selectivity for Hepatocytes. We compared transfection results obtained on $\rho(+)$ and $\rho(-)$ cells with neutral PEI-gal/DNA complexes in simultaneous experiments to provide an idea of the targeting efficiency in conditions relevant to *in vivo* transfection. A fair comparison should include cells transfectable to similar extents with nontargeted vectors in serum. As shown above, BNL CL.2 [$\rho(+)$], HepG2 [$\rho(+)$], and 3T3 cells [$\rho(-)$] fulfill this condition. With regard to the charge of the PEI-gal/DNA complexes, electrophoresis showed that most of the DNA remained in the well when $N/P = 2$ and that the residual faint smear disappeared when $N/P = 3$ (Figure 2). As discussed in the previous paragraphs, this and surface charge measurements are good evidence for the formation of slightly anionic and neutral complexes at these ratios, respectively.

Transfection of BNL CL.2 and HepG2 hepatocyte cell lines with PEI-gal/pCMV-Luc complexes at $N/P = 3$ and 2 was very efficient [0.6 – 1×10^9 LU/(mg of protein)];

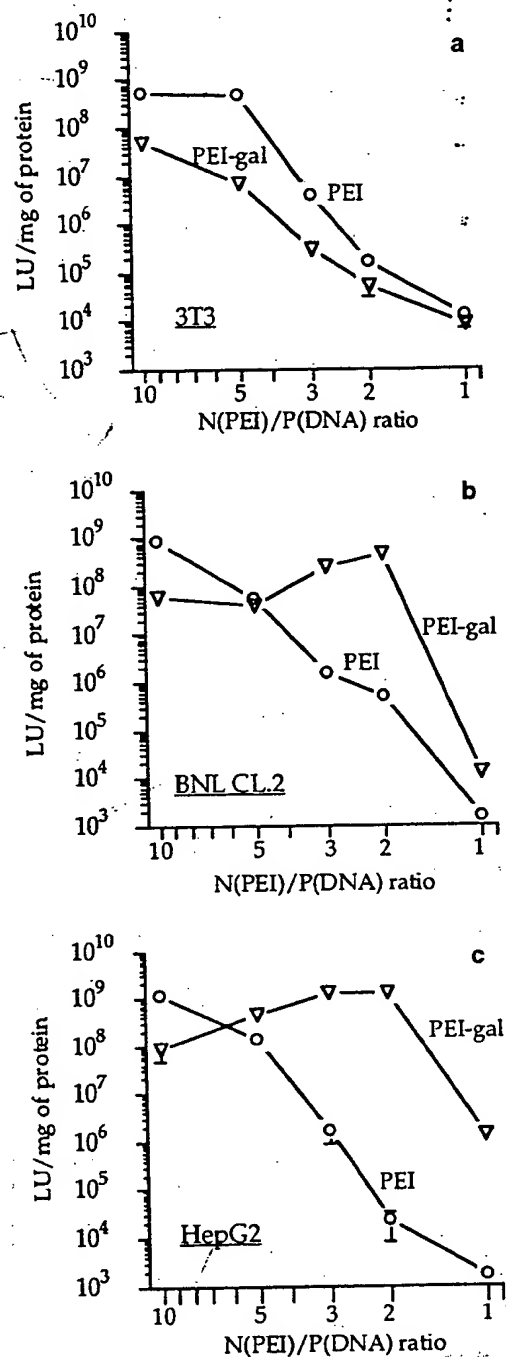


Figure 3. Graph of transfection efficiency vs vector/DNA ratio. 3T3 fibroblast (a), BNL CL.2 (b), and HepG2 (c) hepatocyte cell lines were transfected in the presence of 10% serum with 2 μ g of pCMV-Luc plasmid/well and the desired amount of PEI and PEI-gal as described in Experimental Procedures. Values are the mean \pm SD of three experiments. For most experiments, circles and triangles on the graphs were larger than standard deviations.

transfection of 3T3 fibroblasts was 10^4 – 10^5 -fold less efficient (Figure 4). This very large factor was confirmed histochemically following transfection with the pCMV-NLSLacZ reporter gene; BNL CL.2 cells showed >50% blue nuclei (Figure 5), whereas 3T3 cells hardly showed any (0–2 cells/well, i.e. 0–0.002%, not shown).

Transfection of Hepatocytes Is Receptor-Mediated. The external membrane of hepatocytes is rich in receptors that bind and internalize terminal galactose-bearing asialoglycoproteins. Preliminary evidence for the involvement of these receptors in the gene delivery process was inferred from the high level of reporter gene

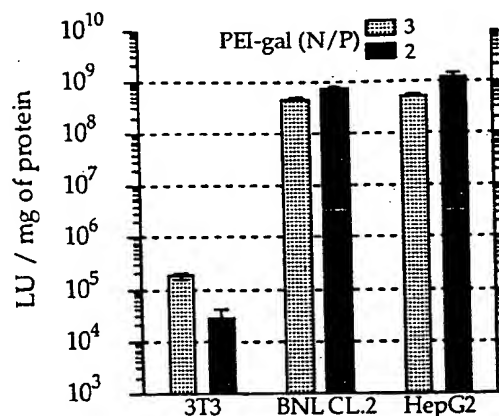


Figure 4. Neutral galactose-bearing complexes deliver genes selectively to hepatocytes. 3T3 fibroblast and hepatocyte (BNL CL.2 and HepG2) cell lines were transfected in a single run with 2 μ g of pCMV-Luc and PEI or PEI-gal (N/P = 2–3) as described in Experimental Procedures. Values are the mean \pm SD of three experiments.

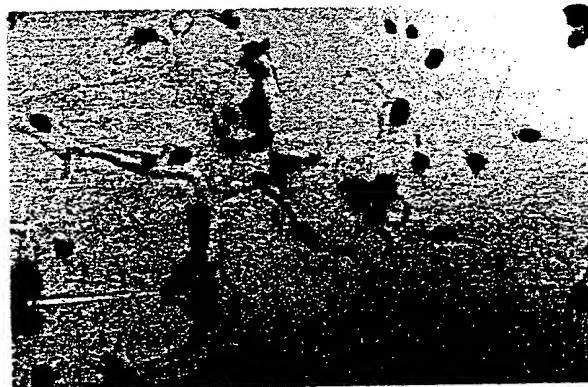


Figure 5. Transfection of BNL CL.2 hepatocytes with neutral PEI-gal/DNA complexes is very efficient. Cells were transfected in 10% serum with PEI-gal and 2 μ g of pCMV-NLSLacZ at N/P = 2. After 24 h, cell permeabilization and histochemical staining with X-gal revealed >50% NLSLacZ-positive cell nuclei. Control experiments at N/P = 2 performed with PEI on BNL CL.2 cells and with PEI-gal or PEI on 3T3 cells showed 0–2 blue nuclei per 10^5 cells in a well.

expression that was observed with neutral PEI-gal/DNA complexes (see above and compare experiments 1 and 6 with experiment 11 in Figure 6).

Additional evidence came from the following comparisons. Removal of the targeting element (galactose) present on the complexes resulted in complete loss of transfection for $\rho(+)$ cells (Figure 6, experiments 2 and 7 vs 1 and 6); no such change was observed for $\rho(-)$ cells (Figure 6, experiment 12 vs 11). However, a glycosylated vector may have other properties affected besides the targeting phenomenon. We therefore synthesized a 5% glucose-bearing polyethylenimine (PEI-glu) by reacting the disaccharide maltose with PEI under the conditions described for PEI-gal. Transfection of 3T3 cells with PEI-glu was negligible and close to that obtained with PEI-gal (experiments 11 and 13); with hepatocytes, however, transfection became 10000- and 100000-fold less effective (experiments 1 and 6 vs 3 and 8). Finally, asialofetuin (ASF), a natural glycoprotein ligand of the receptor, was added to the cell culture medium during transfection (1 mg/well). This protein was not toxic to the cells, nor did it interfere with receptor-independent transfection (Figure 6, experiments 4 and 9). However, competition between PEI-gal/DNA complexes and ASF (36 molar equiv excess with respect to galactose) decreased transfection to very low levels (Figure 6, experi-

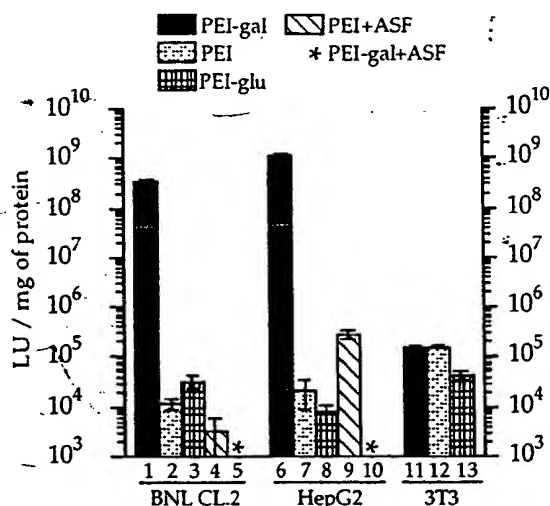


Figure 6. Comparative experiments favoring a receptor-mediated gene delivery process. Cells were transfected with PEI-gal, PEI, or PEI-glu complexed to 2 μ g of pCMV-Luc DNA at N/P = 2 in the presence of 10% serum. Luciferase activity was important only for hepatocytes with PEI-gal. Addition of asialofetuin (ASF at 1 mg/well, 36 molar equiv with respect to galactose) 10 min before addition of the complexes abolished transfection in this case also [* is $<10^3$ RLU/(mg of protein)].

ments 5 and 10). Taken together, data from comparative and competitive experiments all point to the involvement of the asialoglycoprotein receptor in hepatocyte transfection with PEI-gal/DNA complexes.

DISCUSSION

Hepatocytes are interesting as target cells for several reasons. The asialoglycoprotein receptor they express is well-characterized and abundant, and its interaction with a ligand leads to endocytosis. Furthermore, the liver is the center organ of several genetic, acquired, and viral diseases amenable to gene therapy (Frese et al., 1994; Ledley, 1993). Successful *in vitro* targeting of hepatocytes has been demonstrated in many cases, with variable (10^1 – 10^2 -fold) selectivities and experimental conditions (Plank et al., 1992; Midoux et al., 1993; Cristiano et al., 1993; Merwin et al., 1994; Chen et al., 1994; Remy et al., 1995; Wadhwa et al., 1995). In our experimental setup, PEI-gal is more selective, with factors in the range of 10^4 – 10^5 -fold. Due to its endosome-buffering property, PEI-gal is also intrinsically more efficient than polylysine-based vectors which require chloroquine (Plank et al., 1992; Midoux et al., 1993; Merwin et al., 1994; Wadhwa et al., 1995), fusogenic oligopeptides (Plank et al., 1992; Midoux et al., 1993), or adenoviral particles (Plank et al., 1992; Cristiano et al., 1993) to be efficiently released in the cell cytoplasm (all these additives would cause either toxicity or immunological problems *in vivo*). Indeed, with neutral complexes and although cells were maintained in 10% serum, over 50% of hepatocytes were transfected, corresponding to 10^9 LU/(mg of protein) with the luciferase reporter gene; these values fell to 0.002% and 5×10^4 LU/(mg of protein) in the absence of the receptor, respectively. Receptor-mediated transfection in our conditions is thus very close to an all-or-nothing process; most of the 10^5 ρ (+) cells in a well [whereas only a few ρ (-) cells] are transfected, leading to the extreme range found above. This interpretation is in agreement with a "quantum" transfection when strongly driven reporter genes are used, as discussed earlier (Boussif et al., 1996).

Other attractive features of this delivery system in relation to targeting of the liver are consequences of its

electroneutrality. The particles should not bind to extracellular matrix anionic proteoglycans, and neutral PEI/DNA complexes have also been shown to have reduced complement activation properties (Plank et al., 1996). Yet on the extreme sides of the fate of a therapeutic plasmid, i.e. complex formation with the vector and nuclear membrane crossing, the most challenging problems remain unsolved. (i) Downsizing of the particles to avoid Kupffer cells and to cross the vascular endothelium fenestration is one problem. Gene transfer *in vivo* with small polylysine-gal/DNA complexes has been reported (Perales et al., 1994); unfortunately, preliminary transmission electron microscopy pictures of PEI-gal/DNA complexes show rather large and polydisperse 100–400 nm particles. (ii) Restricted cytoplasmic diffusion (whether concerning the PEI/plasmid complexes or the free plasmid) and nuclear membrane crossing are the main barriers to gene delivery with cationic vectors (Labat-Moleur et al., 1996; Zabner et al., 1995). Whereas no satisfactory solution for crossing the nuclear membrane has been described so far, hepatocytes may provide a solution to intracellular trafficking. Indeed, in contrast to the forced and "unnatural" endocytosis process of cationic vector/DNA particles (Labat-Moleur et al., 1996), receptor-mediated DNA uptake into hepatocytes with PEI-gal may lead to endosomes which are able to travel along microtubules toward the centrosome, which is located very close to the nucleus (Novikoff et al., 1996).

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